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Invited review

The use of gene knockout mice in thermoregulation studies

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Abstract

As the use of gene knockout models in thermoregulation studies has gained popularity, the reported incidence of redundant or discrepant phenotypes between studies has also increased. Several gene knockout models mimic human processes and have provided valuable insight into the role of endogenous mediators in thermoregulatory processes. There are also many examples of mutant strains expressing virtually identical phenotypes as their wild-type controls, causing concern regarding the appropriateness of these models for the study of physiological processes. In some cases, discrepancies in results are being reported from different laboratories that are studying the same gene knockout model. While mutant strains provide a powerful tool for analysis of gene function in vivo, the breeding strategies used to generate the strain may have a profound impact on the expressed phenotype. This review examines the intricacies of working with a small rodent such as the mouse and discusses the advantages and disadvantages of using gene knockout models for thermoregulatory research. A number of experimental strategies that can be used to minimize the occurrence of redundant phenotypes are presented. The influence of background strain effects is also considered, since this may be one of the most important factors influencing a mutant phenotype. A future perspective is provided in which more advanced technologies using conditional gene inactivation and the production of rat knockout strains will improve current experimental design.
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Keywords: Temperature regulation; Targeted mutagenesis; Cytokines: Fever; Interleukin-1; Interleukin-6; Mutant mouse models

Contents

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1. Introduction

The advent of targeted mutagenesis of the mouse germ line represents an important breakthrough since it is now possible to study animal populations with mutations of practically any gene of interest. While many gene knockout mouse models successfully mimic responses observed in human disease (i.e., hypothermia, fever), the appearance of an unexpected or redundant phenotype has resulted in controversy concerning the appropriateness of these models in the study of thermoregulatory processes. In addition, the development of gene targeting strategies predominantly in mice, rather than rats, has limited the application of the technology to this species.

It is clear that gene targeting mutagenesis is here to stay. Rapid advances in the technology are improving experimental design and the recent introduction of rat mutagenic models is allowing an expansion of genetic manipulations into this species. This may result in a rapid increase in the use of gene knockout models since the rat is considered by many to be the preferred species for thermoregulatory studies with its more docile nature and ease of manipulation (e.g., instrumentation). However, the mouse still represents the most commonly utilized species for the production of mutant strains. It is important to understand the limitations and specific requirements of working with the mouse to fully utilize the advantages offered by mutant strains for the study of thermoregulatory processes. It is also important to be aware of the limitations of knockout models such that past mistakes in their use are not repeated. This review describes the unique characteristics of the mouse as a model for thermoregulation and discusses the advantages and disadvantages inherent in the use of gene knockout models (mouse or rat). Potential solutions to overcome the limitations of this technology are presented along with a future perspective focused on the recent generation of rat mutagenic models.

2. Homologous recombination for the generation of mouse mutant strains

Provided here is a brief description of the most common procedure used to generate gene knockout strains, termed homologous recombination. The reader is referred to several reviews on the topic for a more detailed description of this technique (Bockamp et al., 2002; Melton, 1994; Muller, 1999; Van der Weyden et al., 2002).

The basic strategy for producing genetically modified mice is homologous recombination (i.e., recombination of a genomic DNA fragment with an endogenous homologous sequence) using mammalian embryonic stem (ES) cells. Fig. 1 provides an outline of this technique for the generation of null mutant mice. ES cells represent pluripotent derivatives of the inner cell mass of the mouse blastocyst. The first step in the production of a null mutant mouse is construction of the targeting vector. The targeting vector contains a sequence homologous to the targeted gene that will recombine with and mutate a specified chromosomal locus. An essential component of this targeting vector is the inclusion of a positive selection cassette, which confers antibiotic resistance. The targeting vector typically consists of the selection cassette (i.e., neomycin) flanked by coding exons (gray boxes) that serve as the target sites for the recombination event. The vector is linearized and electroporated into cultured ES cells with successful homologous recombination determined by positive selection for a neomycin analog (e.g., G418) which confers resistance (as provided by the selection cassette). Positively selected ES cells are injected into a mouse blastocyst and implanted into the uterus of a pseudopregnant foster mother for the production of chimeric pups. Based on this strategy, the genetic makeup of the mouse pups is a combination of cells from the mother's blastocyst and derivates of the targeted ES cells.

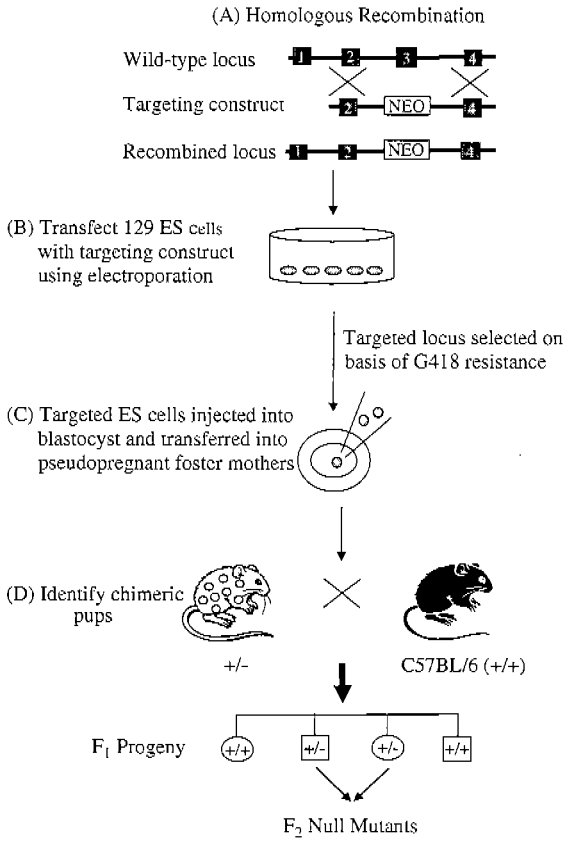

Fig. 1. Procedure for the generation of null mutant mice by homologous recombination in ES cells. (A) Homologous recombination strategy used to generate a knockout genotype. Four exons of the wild-type locus (gray boxes) recombine with the targeting construct, which introduces the selection cassette and deletes exon 3. (B) ES cells are isolated from the inner cell mass of blastocysts from 129 strain of mice and are transfected with the recombined locus using electroporation. (C) Homologously recombined ES cells (determined by positive selection using neomycin resistance) are injected into a blastocyst, which is then transferred to a pseudopregnant foster mother. (D) Chimeric mice are identified and, in many cases, backcrossed to C57BL/6 mice to generate F_1 progeny. Heterozygous F_1 progeny are identified and mated to produce F_2 null mutant mice.

For reasons not completely understood, ES cell lines from the 129 mouse strain are the most compliant for manipulation in culture and repopulation into the germ line. However, due to poor reproductive performance of 129 strains, chimeric pups are generally backcrossed to a different inbred strain for the production of homozygous mutants (Fig. 1). In most cases, investigators choose females of the C57BL/6 strain for breeding to 129-derived male chimeras. In addition to providing more rapid, robust reproductive performance, the use of C57BL/6 females permits monitoring of germ line transmission using coat color markers (Muller, 1999). Based on this strategy, heterozygous F_1 progeny are generated, which contain one set of chromosomes from each parental strain (50% from 129 and 50% from C57BL/6). Intercrossing of F_1 animals is then performed to generate mice homozygous for the mutation along with their wild-type littermates. These offspring represent a random mix of the parental (C57BL/6 and 129) chromosomal DNA throughout their genome.

3. Unique characteristics of the mouse model

> A mouse may be treated as a "test tube" for a molecular or biochemical experiment, but for a complex trait, it must be treated as a complex, living organism". M.P. Printz, 2004

The mouse is considered an excellent model for experimental gene studies due to its relatively short life cycle, its genetic similarity to humans and the ability to easily manipulate its genome. However, despite their genetic similarities, there are large physiological and behavioral differences between mice and humans that need to be considered for effective extrapolation of results between these species.

3.1. Impact of ambient temperature

One facet of rodent research that often confounds data interpretation is the impact of ambient temperature (T_a) on thermoregulatory processes. Historically, the profound impact of T_a on the thermoregulatory capacity of mice and rats has been ignored, with the choice of housing T_a commonly dictated by the comfort of laboratory personnel (typically 20–22 °C). The housing of rodent species under strict environmental conditions is often compromised due to the high cost of maintaining precise control over ambient variables, such as temperature, humidity and lighting. As a consequence, needs of the particular species or strain under study are secondary, with neglect for the impact of T_a on study outcome.

The ability of mice to maintain T_c within a narrow range despite large fluctuations in T_a does not imply that T_c is independent of environmental temperature. On the contrary, the maintenance of normal T_c, particularly under conditions of low T_a, requires significant expenditure of energy. This is particularly true of the laboratory mouse due to its relatively large surface area to body mass ratio (SA:M_b), which facilitates body heat loss to the environment. The housing T_a of small rodents, particularly mice, may have a marked effect on several processes including metabolic rate, food and water consumption, body weight maintenance, locomotor activity and immune

responses, which in turn may influence T_c control processes. For example, housing at 22 °C induces ~2-fold increase in metabolic rate in mice compared to housing at thermoneutrality of 33 °C (Herrington, 1940). Swoap et al. (2004) showed a linear decrease in heart rate, mean arterial pressure and metabolic rate with increases in T_a (18–30 °C) for the mouse and rat. Not surprisingly, due to an ~10-fold difference in body size, mice showed a greater responsiveness to T_a effects on metabolic rate and cardiovascular variables than the rat (Swoap et al., 2004). It has been shown in several species that circadian amplitude increases with a decrease in T_a due to a reduction in daytime T_c at lower housing temperatures (Refinetti, 1997). Given that most thermoregulation studies are performed during the period of the circadian T_c nadir (i.e., lights-on period) in rodents, differences in T_a, and its subsequent effects on T_c amplitude, metabolic rate and other physiological processes could have a large impact on study results and interpretation.

T_c maintenance in rodents is achieved through several mechanisms, including shivering thermogenesis, nonshivering thermogenesis in brown adipose tissue (BAT), changes in vasomotor tone, and behavioral thermoregulation. When provided the opportunity, rodents use behavioral thermoregulation preferentially due to the energy-conserving properties of this strategy over shivering and nonshivering thermogenesis (Gordon, 1983; Kozak et al., 1994; Morimoto et al., 1986; Schmidt, 1984). Under most experimental conditions, the only behavioral means provided for T_c maintenance are group housing and/or cage bedding, which permit heat conservation through huddling and nesting behavior, respectively. Interestingly, even cage bedding, which may be considered a minimal housing requirement that is sufficient to override the effect of low T_a, has been shown to have a differential effect on mouse T_c and metabolic rate depending on the material provided (Gordon, 2004). Heat-treated wood shavings, which provide the best opportunity to burrow and reduce heat loss, was associated with a higher daytime T_c (Gordon, 2004). These effects of housing environment can have a profound effect on thermoregulatory homeostasis and rodent health and well-being.

While wild-type strains have the metabolic efficiency to compensate for body heat loss under conditions of low T_a, the assumption that gene knockout strains will similarly adapt to a mild cold stress (i.e., low housing T_a) may not be a valid assumption (and in most cases has not been experimentally tested). This is illustrated by the most widely recognized mutant strain, the obese *(ob/ob)* mouse. The *ob/ob* mouse was discovered at the Jackson Laboratories in 1950 as a C57BL/6 strain that incurred a spontaneous mutation resulting in an obese phenotype (Ingalls et al., 1950). It was the rarity of the obese phenotype in typical mouse populations that made the recognition of this mutation readily apparent (Ingalls et al., 1950). In addition to its robust obese phenotype, which makes this strain perhaps the most widely recognized mutant mouse strain today, the *ob/ob* mouse also manifests several characteristics of the diabetic syndrome, including hyperglycemia and hyperinsulinemia. With respect to its thermoregulatory capabilities, the inability to survive exposure to 4 °C demonstrated defective thermogenesis in this species, which is now known to be due to decreased thermogenic capacity of BAT (Davis and Mayer, 1954; Hogan and Himms-Hagen, 1980; Trayhurn and James, 1978). While 4 °C represents a significant thermal stress, housing at T_a of 10, 15, 20, 25, and even 30 °C, the latter of which might be considered within the thermoneutral zone (TNZ) of mice, induced hypothermia in *ob/ob* mice compared to their lean littermates (Trayhurn et al., 1976). It was subsequently shown that the preferred T_a of *ob/ob* mice, when allowed to behaviorally select T_a in a thermal gradient, was significantly greater (25–35 °C) than that of their lean littermates (<25 °C). Interestingly, availability of behavioral thermoregulation was not sufficient to correct their hypothermia, suggesting a lowered thermal setpoint in the *ob/ob* strain (Wilson and Sinha, 1985). We now know, due to its discovery in 1994 (Zhang et al., 1994) that the circulating protein leptin, which is encoded by the *obese* gene, is the mutated factor in the *ob/ob* strain that is responsible for several of its physiological abnormalities. The stimulating effect of leptin on metabolic rate and fuel availability are now well recognized (Pelleymounter et al., 1995) and illustrate the potential impact of a gene's mutation on physiological processes that are not directly related to thermoregulation, but can have a profound impact on T_c homeostasis.

3.2. Importance of biotelemetry

Prior to the advent of biotelemetry, which permits the remote sensing of physiological variables (e.g., T_c, ECG) throughout the circadian cycle, T_c was measured using rectal probes. Rectal probes are typically used in conjunction with anesthesia and/or physical restraint, which compromises thermoregulatory control mechanisms by preventing behavioral and physiological responses that may be critical for the manifestation of a normal T_c response in rodents (Hanagata et al., 1995; Stoen and Sessler, 1990). Due to the need for restraint, rectal probes only permit analysis of T_c at discrete times during the circadian cycle, typically during the lights-on or inactive period, which is most convenient for laboratory personnel.

With preference of the mouse toward behavioral mechanisms of T_c regulation, biotelemetry is considered the method of choice for thermoregulation studies in this species. Using biotelemetry, the circadian T_c and

activity rhythm of rodents is typically described as a biphasic pattern consisting of low daytime and high nighttime values that reflect the nocturnal behavior of the species. As a population, C57BL/6 mice display a low daytime (12 h average, 35.99±0.07 °C) and high nighttime (12 h average, 37.27±0.06 °C) circadian T_c rhythm that is mirrored by changes in general locomotor activity. A characteristic of this mouse rhythm that is generally ignored is the manifestation of ultradian fluctuations in T_c and activity that are displayed by individual animals throughout the 24-h cycle. As shown in Fig. 2A, when the T_c profile of a single mouse is observed, a series of transient, robust T_c fluctuations of 1–2 °C occur throughout the circadian cycle. These T_c fluctuations are associated with concurrent spikes in activity and are representative of what has typically been described as rest-activity cycles for this species (D'Olimpio and Renzi, 1998; Poon et al., 1997; Szelenyi et al., 2004; Weinert and Waterhouse, 1998). While group housing or housing in small cages attenuates the magnitude of these ultradian fluctuations (Crowley and Bovet, 1980; Poon et al., 1997), individually housed mice (a condition that is required with the use of biotelemetry) display these fluctuations at different time points during the circadian cycle, with a frequency that has been estimated as ranging from 9 to 20 min (D'Olimpio and Renzi, 1998). Perhaps, what has not been as universally recognized is the potential impact of these ultradian T_c fluctuations on the magnitude of an experimentally induced T_c response. As shown in Fig. 2A, a delta T_c response elicited in a mouse starting at point *b* (~35.5 °C) and reaching a maximum T_c of 38.0 °C will be significantly greater than that elicited from point *a* (~36.8 °C) in the ultradian cycle. These ultradian fluctuations do not appear to be T_a dependent since they are of virtually identical magnitude in mice housed at 25 and 30 °C (unpublished observations). To eliminate the confounding influence of ultradian rhythmicity on an elicited response, it is necessary to examine individual mouse thermoregulatory profiles (rather than group profiles) to determine the most appropriate baseline T_c value before introducing a stimulus in this species. Interestingly, rats do not show these robust rest-activity cycles in their T_c and activity rhythm (Fig. 2B). This is likely due to the significant difference in SA:M_b, lower reliance on BAT and/or lower metabolic rate of this species, which does not support such rapid fluctuations in heat storage.

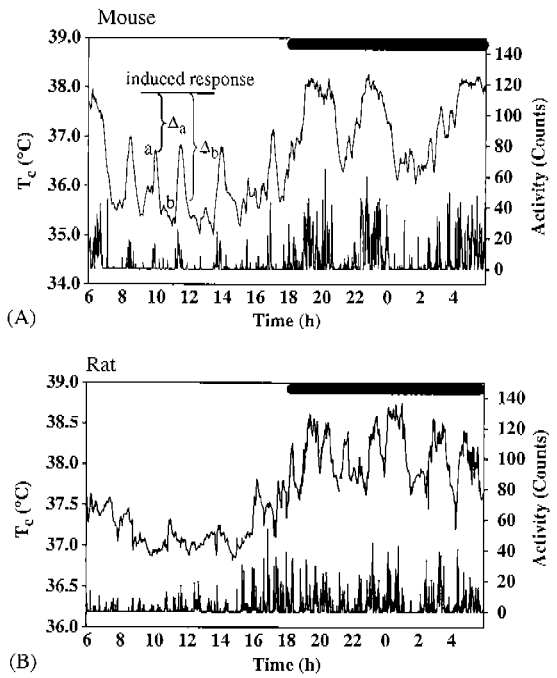

Fig. 2. Circadian rhythm in core temperature (T_c) and motor activity (measured by biotelemetry) of a C57BL/6J male mouse (top) and a Sprague–Dawley male rat (bottom) housed at 25 °C. Data represent 1-min values collected in conscious, unrestrained animals over a 24-h cycle. Dark horizontal bar at the top of each graph represents the lights-off, or active period on a 12:12 h light:dark schedule (lights on at 0600 h). Both animals display a profile with low daytime and high nighttime T_c values that are mirrored by changes in motor activity. The C57BL/6J mouse shows 1–2 °C ultradian fluctuations in T_c that are not observed in the rat. The potential impact of these ultradian T_c spikes on an elicited thermoregulatory response in the mouse is depicted. The delta T_c response elicited in a mouse starting at point *b* (~35.5 °C) and reaching a maximum T_c of 38.0 °C is significantly greater (see Δ_b) than that elicited from point *a* (~36.8 °C; see Δ_a) in the ultradian cycle.

One of the most important considerations in the use of biotelemetry for T_c measurements in small rodents is the impact of the transmitter device on the normal physiological functioning of the animal. With an ~10-fold difference in body size between the mouse and rat, it is expected that intraperitoneal implantation of a biotelemetry device would have a differential effect on growth of these two species. The typical transmitter designed for use in the mouse and rat weighs ~3.6 g, which represents ~12–18% and ~1–2% of mouse (20–30 g) and rat (250–300 g) body mass, respectively. Typically, expression of a robust circadian T_c and activity rhythm following surgical implantation is regarded as evidence of surgical recovery from the intraperitoneal implantation of the transmitter. However, we recently demonstrated that implantation of these transmitter devices in a mouse significantly retards post-surgical growth compared to the rat (Leon et al., 2004). While the rat requires ~1 week for recovery of pre-surgical body weight following transmitter surgery, the mouse requires ⩾14 days. For unknown reasons, water consumption never returned to pre-surgical levels in implanted mice. Thus, while biotelemetry is recommended for studies of thermoregulation in rodents, due

to the elimination of stress-induced alterations in T_c, the elimination of restraint and anesthesia, and the ability to monitor physiological variables throughout the circadian cycle, the mouse presents a unique challenge in the application of this technology due to its small body size. It is recommended that circadian rhythmicity be used as only one of several criteria to indicate surgical recovery prior to inclusion of small rodents in a biotelemetry study. One must consider not only the surgical site of implantation, but also the size of the transmitter device to determine the potential impact on post-surgical growth and normal physiological processes. As such, it is recommended that the manifestation of a robust circadian T_c and activity rhythm be used in conjunction with a recovery of pre-surgical BW to ensure that the device alone does not impact study outcome (Leon et al., 2004).

3.3. Thermal preference

We are entering an advanced stage in the use of mutagenic technology in which a myriad of gene mutant strains are being created or are commercially available for virtually any genetic process that we wish to study. Unfortunately, we still have very little data regarding the impact of mutagenic changes on normal thermoregulatory processes of mice. For example, we have amassed little data regarding changes in the homeostatic physiology of each gene knockout mouse studied. In most, if not all cases, wild-type and gene knockout mice are housed in identical environmental conditions. Is this appropriate?

The TNZ is defined as the T_a range equivalent to minimum metabolic rate and at which T_c is maintained by nonevaporative physical processes (IUPS, 2001). This has been determined as 30–32 °C in several strains of wild-type mice when they are allowed to use behavioral means to select a wide T_a range following placement in a thermal gradient (Hart, 1950; Herrington, 1940; Hudson and Scott, 1979; Mount, 1971). The TNZ has only been determined for select wild-type strains and it is unknown how these values correlate to the wide-variety of wild-type and gene knockout strains that are commercially available today.

My laboratory recently compared the circadian T_c profile of C57BL/6J and B6129F$_2$ (C57BL/6 × 129) male mice (recommended control strains for several gene knockout models provided by Jackson Laboratories) in a thermal gradient. Using biotelemetry to measure T_c, the two strains of mice showed similar 12 h daytime (36.19 ± 0.23 °C) and nighttime T_c values (36.68 ± 0.23 °C). The B6129F$_2$ strain appeared to have slightly higher daytime and nighttime T_c, but this did not represent a significant difference. However, there was a significant difference in the thermal preference of these two wild-type strains (Fig. 3). While B6129F$_2$ mice

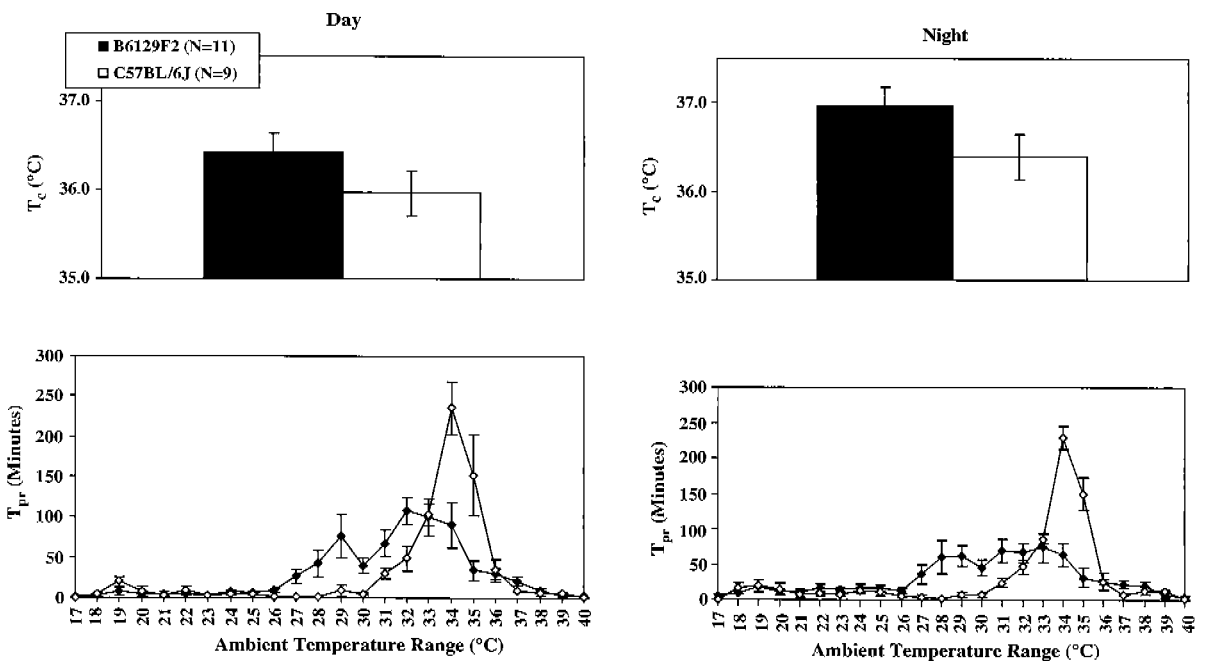

Fig. 3. (A) Core temperature (T_c; biotelemetry) and (B) thermal preference (T_{pr}) of B6129F$_2$ and C57BL/6J male mice during 24 h in a thermal gradient that ranged in ambient temperature from 17 to 40 °C. T_c was collected at 1-min intervals and averaged into 12-h bins to determine day and night values. T_c was calculated as minutes spent at each ambient temperature along the length of the gradient over the 12 h time periods. Food and water were provided along the length of the gradient.

preferred a wider range of T_s ranging from 27 to 36 °C, C57BL/6J mice spent the majority of time residing at 34–35 °C. These ranges of values differ markedly from previously reported values for the mouse (Eedy and Ogilvie, 1970; Gordon, 1985; Ogilvie and Stinson, 1966). While it is generally assumed that SA:M_b is the defining factor for thermal preference, thus predisposing laboratories to house mice of different strains in identical environmental conditions, these data suggest that the genetic background of different wild-type strains may have a previously unrecognized direct impact on thermoregulatory processes.

Despite scant data available on the thermal preference of different wild-type strains, a consideration of the impact of T_a on thermoregulatory responses of gene knockout strains has virtually been ignored (with the exception of *ob/ob* mice, in which cold tolerance has been extensively studied; Ohtake et al., 1977; Qiu et al., 2001; Trayhurn et al., 1976; Wilson and Sinha, 1985). It is generally assumed that a TNZ of 30–32 °C, which is typically described for wild-type strains, is also applicable for gene knockout mice. A consideration of potential differences in thermal preference may be particularly important for those gene products that have been directly implicated in thermoregulatory control. Importantly, potential differences in the thermal preference of wild-type versus gene knockout mice will determine how T_c responses to a variety of inflammatory and environmental stimuli are interpreted between these populations.

4. Advantages and disadvantages of gene knockout models

The low cost, docile nature, well-defined genetic information, and ease of husbandry of the mouse (*Mus*) and rat (*Rattus*) are several reasons for the use of these species in the study of T_c control mechanisms. Historically, the rat has been one of the most widely used species for thermoregulation studies. Decades of experimental study in this species have delineated the cellular and molecular determinants of T_c responses during health and disease.

Traditionally, three experimental approaches have been used to study a protein's role in a thermoregulatory process. These include: (1) application of the protein at the hypothetical site of action to induce a T_c response, (2) measurement of release of the endogenous protein during a thermoregulatory event, and (3) in vivo elimination of the action or production of the protein to alter a T_c response. While the first two methods provide evidence in favor of a role of the targeted protein in a T_c response, only through elimination of protein action/production is the endogenous function of a substance delineated.

While the injection of antibodies or protein inhibitors is a straightforward method to neutralize an endogenous protein, there are several technical difficulties inherent in drug application studies (Table 1). Determination of the anatomical site for injection/action may be difficult, although in most cases the hypothalamus, which represents the thermoregulatory control center, would be an appropriate site to target. Once an anatomical site has been identified, limited access to that site may complicate the injection procedure. While many, if not all, brain sites are accessible through direct cannulation, there are technical difficulties involved in targeting the anatomical site on both sides of the brain. The unknown or limited protein half-life and biological distribution of a compound complicates interpretation of negative results. It is often difficult to determine if the lack of an effect of an injected substance is due to improper dose, rapid breakdown of the product following injection (such that it is not active at the required time of the response) or inadequate distribution to the site where it is required to act. The latter concern is always a consideration for substances that are injected peripherally and expected to cross the blood-brain barrier in order to induce a response. Additional concerns include undesired nonspecific or cross-reactivity effects, undesirable stress effects from the injection procedure and/or lack of commercial availability of the drug. Similarly, the ability to study long-term effects of a protein's absence is limited by these difficulties (Yap and Sher, 2002).

Gene knockout mice, which have been engineered to lack a gene product throughout development and in all tissues, may be viewed as "chronic protein

Table 1
Advantages and disadvantages of gene knockout models

Advantages
Chronic protein neutralization systems
Eliminates drug delivery complications
Appropriate injection/infusion site
Short half-life
Limited or unknown distribution
Specificity/cross-reactivity
Stress effects due to handling
Lack of commercial availability
Disadvantages
Selection cassette interference
Functional redundancy/compensation
Lack of developmental/tissue specificity
Lethal knockouts/survival of the fittest
Differences in background genotype/hybrid strains
Substrain variability (e.g., 129)
Hitchhiker/modifier genes
Proper wild-type controls
Strain specific responses
Hybrid vigor (e.g., FVB/N)

neutralization systems" (term adapted from Sigmund, 1993). As such, they are advantageous for protein analysis in a thermoregulatory response due to the elimination of several of the limitations described for an injected substance (Table 1). The issues of dose, timing of injection and distribution are effectively eliminated with the use of these mouse models. However, it is naive to assume that these models represent the "holy grail" with respect to resolving drug application issues, since as will be discussed below, there are several potential disadvantages and perhaps unrecognized difficulties of working with genetically altered animals that may affect interpretation of an expressed phenotype.

4.1. Selection marker interference

The use of the positive selection (usually neomycin) cassette in the targeting vector can induce altered phenotypes that are distinct from the targeted gene mutation (Gingrich and Hen, 2000). This effect is due to disrupted expression of genes that are immediately flanking the gene of interest (Gingrich and Hen, 2000). Wang et al. (1999) demonstrated interference by the neomycin cassette in a null mutant model of dwarfism, in which removal of the cassette produced a different phenotype that more closely resembled the human condition. Several studies have shown selection cassette interference on expressed phenotypes of mutant strains (Fiering et al., 1995; Meyers et al., 1998; Rijli et al., 1994; Sigmund, 1993). In many cases, investigators may not have direct control of construction of the targeting vector as mutant strains are obtained from other laboratories or commercial vendors responsible for generation of the strain. However, it is important to consider the potential impact of the construct on an observed phenotype.

4.2. Embryonic lethality/survival of the fittest

For gene products required during development, the generation of a null mutant model may result in embryonic lethality of the strain, precluding analysis of a phenotype (Bockamp et al., 2002; Thyagarajan et al., 2003; Yap and Sher, 2002). Conversely, continuous inbreeding of F_2 homozygous mice can result in the "survival of the fittest" phenomenon in which deleterious copies of a gene are lost, providing a selective advantage. As described in more detail below (see Section 6), the development of time- and tissue-specific (i.e., conditional) knockout mice will circumvent these issues.

4.3. Functional redundancy/compensation

The most commonly cited disadvantage of using gene knockout models for the study of physiological processes is the absence of a specific phenotype or the expression of functional redundancy (i.e., compensation) of the missing protein's effect (Table 1; Bockamp et al., 2002; Crawley, 1996; Gerlai, 1996; Hanks et al., 1995; Thyagarajan et al., 2003; Wilder and Rizzino, 1993; Yap and Sher, 2002). One of the most often cited examples of functional redundancy in thermoregulation studies is in the expression of virtually identical circadian T_c rhythms between wild-type and several types of gene knockout strains (Chai et al., 1996; Kozak et al., 1995; Labow et al., 1997; Oka et al., 2003; Zheng et al., 1995). Functional redundancy is expected to occur more frequently in the study of multi-gene families, due to structural and/or functional similarity between family members that may facilitate compensation (Wilder and Rizzino, 1993). One suggestion to avoid redundancy is to construct mice that are doubly deficient in known complementary genes (Yap and Sher, 2002). While simple in theory, in many cases this is not feasible due to the complex network of mediators typically involved in a physiological response that makes identification of the specific genes unlikely. Realistically, the involvement of entire genetic pathways in a response can increase the number of genetic mutations such that this approach is neither feasible nor desirable.

An alternative approach to study the role of gene family members may be to construct mice lacking the functional receptors for that family. For example, the interleukin-1 family consists of several circulating agonists (e.g., IL-1α and IL-1β) and a naturally occurring antagonist (IL-1rn) that function in T_c regulation during inflammation; but there is only one functional receptor (IL-1 type I; IL-1r1) that is capable of transmitting a biological signal (Dripps et al., 1991; Luheshi et al., 1996; Sims et al., 1993). Based on this gene family structure, study of the IL-1r1 knockout mouse is equivalent to studying a mouse lacking all members of the functional IL-1 family of proteins. Of course, the caveat to this approach is that it permits analysis of a protein family but cannot delineate the role of each specific member. Furthermore, there are cytokine receptors which can interact with several ligands from different gene families, such as the IL-6 gp130 receptor which can transmit signals to IL-6 and IL-11, as well as several other cytokines (Benigni et al., 1996). Thus, this approach is not practical for all gene families implicated in thermoregulatory control.

Ideally, one should test a combination of singly, doubly and receptor deficient mice to narrow down the combination of possibilities for a redundancy in a particular gene family. Horai et al. (1998) used this approach to examine the role of the IL-1 family of proteins in the fever response to inflammation. Using a combination of IL-1α, IL-1β, IL-1α/β and IL-1rn knockout mice, they were able to dissociate the roles of these individual proteins in the febrile and

immuno-endocrine responses to turpentine (a local inflammatory stimulus). For a gene family consisting of only one or two members, this is a reasonable approach; however, it may be unrealistic for larger gene families.

It is important to realize that functional redundancy to one stimulus does not imply functional redundancy will be expressed in gene knockout mice to all stimuli with which they are challenged. For example, IL-6 and IL-1r1 knockout mice show virtually identical fever responses as their wild-type controls to a low dose of LPS, but are completely resistant to fever induced by turpentine (Kozak et al., 1998; Leon et al., 1996; Zheng et al., 1995). These responses to LPS in the knockout mice contradict several previously published results supporting a role for endogenous IL-1 and IL-6 in LPS-induced fever (Kluger, 1991). In instances in which functional redundancy contradicts previously established scientific findings, the argument in favor of the use of gene knockout mice for the study of thermoregulatory processes is weakened. However, the data from IL-1 and IL-6 knockout simply indicate that, under the conditions of chronic neutralization of an endogenous signal, functional redundancy exists in the thermoregulatory pathway such that normal responses may still be manifest to a particular stimulus. These findings may have important clinical implications for chronic drug treatment regimens. Therefore, it may be prudent to accept the expression of functional redundancy as important in itself since it provides knowledge regarding alternative biochemical pathways that may otherwise not be recognized (Crawley, 1996).

4.4. Background strain effects

Prior to the wide-availability of gene knockout models from commercial vendors, most animals were created and/or provided by laboratories that generated a null mutant of interest and offered breeding pairs to an interested investigator. This presented researchers the opportunity to study a gene knockout mouse of interest, but resulted in little or no control of the techniques and breeding strategies used to generate the founder population(s). Once breeding pairs are obtained by a laboratory (commercially or otherwise), several strategies are possible to generate a sufficient population for use in a study, but the consequences of the adopted breeding strategy may not be fully realized or documented, making replication of study results difficult.

One of the most influential factors on the phenotype of a gene knockout model is the background strain used to generate the population. ES cells of the 129 strain are most often used for the generation of null mutant mice. Unfortunately, considerable genetic variation exists with respect to the numerous 129 substrains. Simpson et al. (1997) reported a 43% incidence of locus polymorphism among 129 substrains. In some cases, this variation is a direct result of previously unrecognized errors in breeding strategies due to accidental outcrossing that results in genetic contamination of the strain (Simpson et al., 1997; Threadgill et al., 1997). Threadgill et al. (1997) discovered ~20–25% of the 129/SvJ strain to be of non-129 origin, making it the most contaminated 129 substrain. In essence, this strain should be considered a recombinant strain of mixed genetic background, consisting of 129/SvJ and an unknown donor strain, termed "X" (Threadgill et al., 1997). Based on contamination and variability among 129 substrains, nomenclature of this strain was revised to aid in future designation of the strain as it is used for the development of mutant populations (Festing et al., 1999).

Superior reproductive performance and/or hybrid vigor of particular strains are two traits that often favor their use for the generation of mutant populations (Hogan et al., 1986; Taketo et al., 1991). For example, the FVB/N strain is the preferred strain for transgenic analyses due to superior fecundity relative to most inbred strains (Taketo et al., 1991). One must always consider, however, that the background strain of choice may be contributing characteristics which extend beyond their reproductive capabilities, affecting study outcome in an unpredictable or unrecognized fashion. For instance, the mild behavior patterns of the FVB/N, C57BL/6 and 129 strains may make them less than ideal for stress reactivity studies, such as the open field exploration test which is commonly used in the study of stress-induced hyperthermia.

5. Experimental strategies that minimize knockout disadvantages

Provided below are a number of experimental strategies that can be used to overcome the problems of functional redundancy and background strain effects that were described above.

5.1. Breeding strategies to eliminate background strain effects

Maintenance of mutants on a pure inbred (isogenic) strain is the optimal strategy for the elimination of genetic background effects; however, poor performance of the inbred strain in certain experimental paradigms often precludes their use (Muller, 1999; Sigmund, 2000). For example, the 129 strain shows disease susceptibility and poor reproductive performance which deters its use in most breeding strategies. Furthermore, there are little or no data available on the thermoregulatory responses of 129 substrains, making their contribution to study outcome difficult to predict or assess. The use of the C57BL/6 strain for ES cells and chimera breeding is a

reasonable alternative strategy to overcome the unwelcome characteristics of the 129 strain (Gerlai, 1996). The frequent use of the C57BL/6 strain for behavioral and physiological studies facilitates comparison of phenotypes of gene knockout mice to this strain whose responses are already well characterized. This strategy would represent a potential reduction and refinement of animal numbers due to availability of previously described thermoregulatory characteristics in this strain.

The production of congenic strains, achieved by the continuous backcrossing onto a defined (inbred) background strain, is an alternative approach for assessing the contribution of genetic backgrounds to observed phenotypes (Muller, 1999; Sigmund, 2000). Congenic mice are genetically identical to the control strain with the exception of the one chromosomal region of interest. While a simple procedure to perform, the time estimate for generation of congenic strains with >99% homogeneity is ca. 2–3 years, or six generations of backcrossing (Sigmund, 2000), making this a less than desirable procedure for most laboratories. The introduction of "speed congenics" (also referred to as "marker assisted selection breeding") produces congenic strains in as few as five generations, significantly reducing the time investment for congenic strain production (Markel et al., 1997; Wakeland et al., 1997). However, congenic strains are not devoid of background influences since genetic regions from the ES cells that are located adjacent to the targeted locus remain in the mutant animals and may induce effects that are not directly related to the mutated locus (Gerlai, 1996). As estimated by Lathe (1996), 360 or more modifier genes from the ES strain may flank a targeted gene of interest. If these genes have a direct effect on the phenotype being studied, differences between wild-type and null mutant mice may be due to linked or modifier genes rather than the mutation of interest. Qiu et al. (2001) demonstrated the effect of modifier genes on the thermoregulatory phenotype of the *ob/ob* mouse by backcrossing the *ob* mutation for 10 generations from the C57BL/6J to the Balb/cJ genetic background. Improved thermal tolerance to a cold challenge in the Balb/cJ backcrossed mice compared to the original C57BL/6J strain demonstrates the potential effect of background modifier genes on thermoregulatory processes in the *ob* strain (Qiu et al., 2001). It is currently unknown if the modifier genes in the *ob/ob* strain are directly related to the leptin pathway or represent an alternative pathway for thermoregulatory and metabolic control in this model.

Three guiding principles were suggested by geneticists at the Banbury Conference on Genetic Background in Mice (Silva, 1997) to eliminate differences due to background strain effects between studies. Although these recommendations were originally described for the use of transgenic and knockout models in neuroscience, they can be equally applied to all disciplines. The recommendations include: (1) providing enough detail of the genetic background to allow rederivation (or purchase) of the mice by other laboratories, (2) avoiding a complex genetic background that may limit rederivation of the strain, and (3) using a common genetic background to facilitate interpretation between experiments. It is expected that the increase in commercial availability of mutant strains will decrease the need for strain rederivation by individual laboratories, allowing standardization of background genotypes for specific mutant strains. However, in instances in which commercial vendors are used, it is recommended that inclusion of the stock number and specific genotype designation of the mutant and wild-type strains is included in all reports to permit direct comparison between studies. Since the degree of genetic similarity between a congenic and its background strain is dependent on the number of performed backcrosses (Sigmund, 2000), it is also recommended that the backcross generation be included in all reports using mutant strains from commercial or laboratory sources.

5.2. *The importance of the wild-type control strain*

Perhaps the most important consideration for analysis of a mutant phenotype is the wild-type control strain that is chosen for phenotypic comparison. For genetically engineered mice that are maintained on a standard inbred background, the inbred strain itself represents the appropriate wild-type control. When the mutant strain is maintained on a mixed genetic background, the most stringent controls are wild-type mice from the same colony (Muller, 1999; Sigmund, 2000). The use of littermates minimizes environmental variability between populations, as does an analysis of a large number of litters (Bockamp et al., 2002; Gerlai, 1996; Lathe, 1996; Sigmund, 2000). The inclusion of heterozygous littermates, which helps control for genetic background and environmental variability, is a unique strategy that is usually ignored (Bockamp et al., 2002). Typically, the maintenance of mutant populations is achieved through homozygous matings, which eliminates the availability of wild-type and heterozygous littermates as controls. Therefore, in most cases, an exact genetic match of the control to the mutant strain is not possible. Under conditions in which homozygous matings are used for the maintenance of mutant populations, F_2 hybrids are the most appropriate controls (Muller, 1999; Sigmund, 2000). While F_2 hybrids do not represent an exact genetic match to the mutant strain, they contain genes from the two strains used to generate the mutant population. This recommendation follows the guidelines provided by Jackson laboratories, which is a commercial source for several mutant strains (www.jax.org).

As illustrated in the following example, differences in control strain genetics and/or environmental backgrounds can have a significant impact on results interpretation. Using knockout mice from the same source (Zheng et al., 1995), two different laboratories examined the thermoregulatory profile of IL-1β knockout mice to an inflammatory stimulus. Prior to inflammation, the circadian T_c profile of the IL-1β knockout mice was examined to determine if endogenous IL-1β has a role in baseline thermoregulatory processes. The circadian T_c profile of IL-1β knockout mice was reported as elevated (Alheim et al., 1997) or virtually identical (Kozak et al., 1998) to wild-type controls in the two studies. Differences in ambient temperature could not account for these effects since mice were housed at T_a of 30 °C in both studies. Analysis of the fever response to a model of bacterial inflammation (lipopolysaccharide, or LPS, injection) also yielded discrepant results between these studies with IL-1β knockout mice showing enhanced (Alheim et al., 1997) or identical (Kozak et al., 1998) fever responses compared to their respective wild-type controls. Since the same source provided the IL-1β knockout mice, differences in genetic background of the mutant strain was not responsible for these varying results. The use of different wild-type control strains is a likely reason for the phenotypic differences observed between these studies. While one study compared mutant results to those of their wild-type littermates (Alheim et al., 1997), the other study obtained a commercially available wild-type strain (Kozak et al., 1998) whose background may not have adequately represented the genetic and environmental background of the mutant strain. These studies illustrate that, as a general rule, the background genotype needs to remain constant between experiments if phenotypic differences between studies are going to be ascribed to the mutation of interest, rather than to differences in genetic and/or environmental background that may be unrelated to the mutation (Silva, 1997).

5.3. *Rescue experiment*

The strongest evidence that a mutation is responsible for an observed phenotype is when the wild-type phenotype is restored following reintroduction of a functional copy of the gene (Gerlai, 1996; Muller, 1999). There are several experimental techniques that can be used to restore a missing protein's function in a mutant animal including injection or infusion of the missing protein, introduction of a transgene, and the generation of "knock-in" mice (Gerlai, 1996; Muller, 1999).

For mutant strains lacking a circulating endogenous protein (e.g., *ob/ob*), reintroduction of the missing protein using injection or infusion methods is a viable option, with the usual caveat of having to determine the proper dosage and timing of the treatment that will restore endogenous function. Several studies have shown the ability of leptin administration to reverse characteristics of the *ob/ob* mutant phenotype. These effects of leptin injection into the *ob*/ob strain include reduction of food intake and body weight, correction of hyperinsulinemia and hyperglycemia, and increased metabolic rate and body temperature (Gavrilova et al., 1999; Halaas et al., 1995; Harris et al., 1998; Pelleymounter et al., 1995; Weigle et al., 1995). Using a cytokine knockout model, Chai et al. (1996) showed that the peripheral injection of IL-6 was not able to restore the fever response to LPS in mice that lacked the endogenous IL-6 protein (IL-6 knockout mice). These results were interpreted as evidence against peripheral IL-6 being involved in the LPS fever response; however, they only tested the effect of one dose of the cytokine given at a single time point. The ability of centrally injected IL-6 to restore the LPS-induced fever response of the knockout mice was not tested in this study. Thus, this rescue experiment was not successful in showing that the wild-type phenotype could be restored in the mutant mice. However, the ability of centrally injected IL-6 to induce fever by itself was shown and is an important result since it demonstrates that the IL-6 gp130 receptor complex remained functional in the cns of the knockout mice (Chai et al., 1996).

While injection/infusion of the missing protein into a mutant animal is the least technically difficult procedure (although, as previously described, there are inherent difficulties with this procedure), this method is not appropriate under conditions in which a receptor has been ablated, unless the injected receptor is able to reinsert into the cell membrane and function in a manner identical to the endogenous protein. While the soluble IL-6 receptor is known to augment endogenous IL-6 thermoregulatory responses in vivo (Schobitz et al., 1995), to the best of my knowledge this has not been demonstrated for other receptors implicated in T_c control mechanisms. Thus, this may not be an option for thermoregulatory models that use receptor deficient mice. However, an important negative control for experiments using receptor deficient animals is to demonstrate the lack of a response to the injected ligand for that receptor. This is particularly important under conditions in which a redundant phenotype has been observed in the mutant animal. For example, as previously described, IL-1r1 knockout mice develop fever responses to the peripheral injection of LPS that are virtually identical to those observed in wild-type controls (Labow et al., 1997; Leon et al., 1996). These data are controversial since there are abundant data in the literature implicating endogenous IL-1 in the regulation of bacterial-induced fever responses in rodents (Kluger, 1991). The conundrum under these circumstances is whether (1) IL-1 is not an essential regulator of LPS fever since the lack of its type I

receptor (the only known signaling receptor for this cytokine; Sims et al., 1993) has no effect in animals injected with LPS (i.e., other proteins can compensate for its function in the knockout animal), or (2) an as-yet-unidentified receptor for endogenous IL-1 exists in the knockout mice and is responsible for the redundant responses to LPS. To adequately address this question, the ability of IL-1 injection to induce a fever response in wild-type, but not IL-1r1 knockout mice, was a necessary negative control since it verified neutralization of IL-1 actions in the mutant animals (Labow et al., 1997; Leon et al., 1996). It is recommended that a negative control be included in the experimental design of all gene knockout studies that show redundant thermoregulatory phenotypes.

A more advanced method to circumvent drug application issues is to introduce a receptor transgene into the knockout animal to recover the wild-type phenotype (Muller, 1999). The advantage to this technique over drug injection is that it permits analysis of circulating as well as receptor complexes, since the receptor can be targeted for reinsertion into a particular cell type, as required. An interesting variation on this theme is to replace genes using homologues from other species, which may provide a more direct genetic correlate for the study of a human disease process (Moore et al., 1995; Zou et al., 1994). In addition, complementation studies, in which one gene family member has been replaced with another, may facilitate analysis of complex genetic pathways in which multiple gene family members are implicated (Hanks et al., 1995). Finally, the development of "knock-in" mice provides the ideal control for the potential effects of linked or modifier genes. The insertion of a segment of DNA flanking the gene of interest, but not disrupting it, results in a "knock-in" mouse that has the same complementation of genes as the wild-type mouse, but with the addition of the linked genes next to the recombination site that was targeted in the mutant (Gerlai, 1996). This approach allows the contribution of the linked genes to the expressed phenotype to be precisely determined.

Based on the variety of breeding and experimental strategies described in this section, it is clear that the potential for background strain effects and redundant phenotypes complicate the use of gene knockout models in thermoregulation studies. However, using precise record keeping and reporting of breeding strategies used to generate the strain and considering strategies that allow rescue of the mutant phenotype, it is likely that much of the variation between studies can be effectively eliminated. The use of one or several of these strategies would significantly improve experimental design and reduce the complications of working with the variety of mutant strains that are currently available.

6. Future perspective: advancements in mutagenesis technology

6.1. Inducible gene inactivation

One of the major drawbacks of the classical gene targeting strategy is that the gene is missing throughout development and in all tissues of the body. This precludes analysis of a gene's absence at a particular time point during development and does not allow tissue specificity to be determined for a protein's action in a thermoregulatory response. To circumvent these issues, new methods are being developed that allow conditional inactivation of a gene in a time- or tissue-specific manner (Feil et al., 1996; Gu et al., 1994; Kuhn et al., 1995; Muller, 1999; Rajewsky et al., 1996; Seibler et al., 2003). For a more complete review of these techniques and their applications, the reader is referred to several papers on this topic (Bockamp et al., 2002; Kuhn et al., 1995; Muller, 1999; Rajewsky et al., 1996; Seibler et al., 2003; Van der Weyden et al., 2002).

Time-dependent inactivation allows the investigator to activate or inactivate a gene of interest at a particular developmental time point. This is a powerful approach since it allows each animal to serve as its own wild-type control, by comparing the thermoregulatory response pre- and post-induction of the phenotype (Gerlai, 1996). Additional benefits to this strategy include an analysis of genes that would otherwise induce lethal mutations or functional redundancy as their functions are absent during critical stages of development (Gu et al., 1994; Kuhn et al., 1995). On the other hand, tissue-specific gene inactivation permits identification of the physiological role of a protein in a particular tissue while function(s) of the protein in other tissues of the organism remain intact. These models may be particularly useful to model tissue-specific disease conditions that more accurately reflect the human condition than a developmental knockout. To the best of my knowledge, there are no studies examining time- or tissue-specific inactivation of genes that are implicated in thermoregulatory mechanisms.

6.2. Rat mutagenesis

There are several advantages imparted by the rat, over mice that have resulted in their preferred use in thermoregulation studies. The inability to induce fever in mice precluded their use in thermoregulation studies for many years. Although these complications have since been overcome, the fact that many thermoregulatory mechanisms were originally determined in the rat will entice researchers to continue working with this species. The more docile nature of the rat makes it particularly appealing for behavioral studies and for everyday maintenance of the species. Due to its large body size,

instrumentation with physiological devices for measurement of T_c and other physiological variables is better tolerated by the rat. As previously described, the surgical implantation of biotelemetry devices into the peritoneal cavity has a minimal effect on the growth of the rat compared to the mouse, presumably due to the larger size of the rat peritoneal cavity which is better able to tolerate the device (Leon et al., 2004). Sequential blood sampling for the analysis of cytokines and other putative thermoregulatory mediators is more easily provided by the rat whose total blood volume is significantly larger than the mouse. Since most bioassays require 50–100 μl blood samples from each animal, the larger blood volume of the rat permits sequential samples to be collected in each animal. This is an important consideration since sequential sampling of blood variables is a more powerful technique than reliance on single time points, the latter of which can introduce variability between experimental groups. Furthermore, mice afford such small quantities of blood that only a limited number of analyses can be performed on any one animal. Perhaps most importantly, the ~10-fold difference in body size between these species makes T_a effects on heat exchange processes less pronounced in the rat, which is important under experimental conditions in which the TNZ cannot be achieved and/or maintained.

One of the reasons for reliance on mouse knockout models for studies of thermoregulation has been the lack of success of mutagenesis technology in the rat. The inability to produce functional rat embryonic cells precluded the use of this species for the generation of null mutants. However, recent reports indicate that successful methods are now available to induce rat germ line mutations, suggesting that rat knockout models may be available in the near future. Zan et al. (2003) recently reported the successful mutation of breast cancer genes *Brca1* and *Brca2* in Sprague–Dawley rats (a common strain used in thermoregulation research) using *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis. ENU is a germ line mutagen that, when injected into adult rats, induces inheritable mutations (estimated to cause one mutation per 1000 alleles examined; Justice et al., 1999; Noveroske et al., 2000) that can be identified in F_1 offspring to detect mutations of interest. Animals carrying the mutation of interest in the F_1 population are outcrossed to attain homozygosity for phenotypic analysis (Smits et al., 2004; Zan et al., 2003). However, ENU can induce a number of undesired heritable phenotypes, such as ocular and tail abnormalities, a reduction in litter size, infertility and shortened lifespan (Zan et al., 2003). Thus, this technique is not the ultimate solution to the hurdles presented for rat mutagenesis, but it provides one potential mechanism to expand this technology into this species. Recent publication of the rat genome has clearly spurred renewed interest in this species making the availability of new knockout strains more likely in the near future.

7. Concluding remarks

The purpose of this review is to provide insight into the advantages and disadvantages of working with gene knockout models and to recommend experimental strategies that can mitigate many of the undesired consequences of using these models to examine thermoregulatory responses. Although many thermoregulation studies using a variety of gene knockout models have not followed the recommendations provided in this review, that does not imply that their results are without scientific merit. Complications regarding differences in genetic background were not an issue when knockout mice first became available since typically only one laboratory was involved in the production of a mutant strain. However, knockout strains are now commercially available, so "buyer beware"—knowledge regarding the details of strain production and the choice of appropriate controls is the responsibility of the consumer/researcher. In most instances, those of us working in thermoregulation are not trained geneticists, but we are entering an era of working with genetic models that may be indispensable for our understanding of thermoregulatory processes. The recommendations provided here are meant to serve as a general guideline for learning from past mistakes that have been discovered (primarily) in other disciplines that have relied more heavily on this technology. It is hoped that these recommendations will provide a foundation to more thoroughly determine the myriad of factors that may be affecting a mutant phenotype such that the rigidity of future experimental designs in the area of thermoregulatory research is improved.

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References

Alheim, K., Chai, Z., Fantuzzi, G., Hasanvan, H., Malinowsky, D., Di Santo, E., Ghezzi, P., Dinarello, C.A., Bartfai, T., 1997. Hyperresponsive febrile reactions to interleukin (IL) 1$\beta\alpha$ and IL-1β, and altered brain cytokine mRNA and serum cytokine levels, in IL-1β-deficient mice. Proc. Natl. Acad. Sci. USA 94, 2681–2686.

Benigni, F., Fantuzzi, G., Sacco, S., Sironi, M., Pozzi, P., Dinarello, C.A., Sipe, J.D., Poli, V., Cappelletti, M., Paonessa, G., Pennica, D., Panayotatos, N., Ghezzi, P., 1996. Six different cytokines that share gp130 as a receptor subunit, induce serum amyloid A and potentiate the induction of interleukin-6 and the activation of the hypothalamus-pituitary-adrenal axis by interleukin-1. Blood 87, 1851–1854.

Bockamp, E., Maringer, M., Spangenberg, C., Fees, S., Fraser, S., Eshkind, L., Oesch, F., Zabel, B., 2002. Of mice and models: improved animal models for biomedical research. Physiol. Genomics 11, 115–132.

Chai, Z., Gatti, S., Toniatti, C., Poli, V., Bartfai, T., 1996. Interleukin (IL)-6 gene expression in the central nervous system is necessary for fever response to lipopolysaccharide or IL-1β: a study on IL-6-deficient mice. J. Exp. Med. 183, 311–316.

Crawley, J.N., 1996. Unusual behavioral phenotypes of inbred mouse strains. Trends Neurosci. 19, 181–182.

Crowley, M., Bovet, J., 1980. Social synchronization of circadian rhythms in deer mice (*Peromyscus maniculatus*). Behav. Ecol. Sociobiol. 7, 99–105.

Davis, T.R.A., Mayer, J., 1954. Imperfect homeothermia in the hereditary obese-hyperglycemic syndrome of mice. Am. J. Physiol. 177, 222–226.

D'Olimpio, F., Renzi, P., 1998. Ultradian rhythms in young and adult mice: further support for the basic rest-activity cycle. Physiol. Behav. 64, 697–701.

Dripps, D.J., Brandhuber, B.J., Thompson, R.C., Eisenberg, S.P., 1991. Interleukin-1 (IL-1) receptor antagonist binds to the 80-kDa IL-1 receptor but does not initiate IL-1 signal transduction. J. Biol. Chem. 266, 10331–10336.

Eedy, J.W., Ogilvie, D.M., 1970. The effect of age on the thermal preference of white mice (*Mus musculus*) and gerbils (*Meriones unguiculatus*). Can. J. Zool. 48, 1303–1306.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., Chamgon, P., 1996. Ligand-activated site-specific recombination in mice. Proc. Natl. Acad. Sci. USA 93, 10887–10890.

Festing, M.F., Simposon, E.M., Davisson, M.T., Mobraaten, L.E., 1999. Revised nomenclature for strain 129 mice. Mamm. Genome 10, 836.

Fiering, S., Epner, E., Robinson, K., Zhuang, Y., Telling, A., Hu, M., Martin, D.I., Enver, T., Ley, T.J., Groudine, M., 1995. Targeted deletion of 5'HS2 of the murine beta-globin LCR reveals that it is not essential for proper regulation of the beta-globin locus. Genes Dev. 9, 2203–2213.

Gavrilova, O., Leon, L.R., Marcus-Samuels, B., Mason, M.M., Refetoff, S., Vinson, C., Reitman, M.L., 1999. Torpor in mice is induced by both leptin-dependent and—independent mechanisms. Proc. Natl. Acad. Sci. USA 96, 14623–14628.

Gerlai, R., 1996. Gene-targeting studies of mammalian behavior: is it the mutation or the background phenotype? Trends Neurosci. 19, 177–181.

Gingrich, J.A., Hen, R., 2000. The broken mouse: the role of development, plasticity and environment in the interpretation of phenotypic changes in knockout mice. Curr. Opin. Neurobiol. 10, 146–152.

Gordon, C.J., 1983. Behavioral and autonomic thermoregulation in mice exposed to microwave radiation. J. Appl. Physiol. 55, 1242–1248.

Gordon, C.J., 1985. Relationship between autonomic and behavioral thermoregulation in the mouse. Physiol. Behav. 34, 687–690.

Gordon, C.J., 2004. Effect of cage bedding on temperature regulation and metabolism of group-house female mice. Comp. Med. 54, 63–68.

Gu, H., Marth, J.D., Orban, P.C., Mossmann, H., Rajewsky, K., 1994. Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. Science 265, 103–106.

Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., Friedman, J.M., 1995. Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269, 543–546.

Hanagata, K., Matsukawa, T., Sessler, D.I., Miyaji, T., Funayama, T., Koshimizu, M., Kashimoto, S., Kumazawa, T., 1995. Isoflurane and sevoflurane produce a dose-dependent reduction in shivering threshold in rabbits. Anesth. Analg. 81, 581–584.

Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A.B., Joyner, A.L., 1995. Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. Science 269, 679–682.

Harris, R.B.S., Zhou, J., Redmann Jr., S.M., Smagin, G.N., Smith, S.R., Rodgers, E., Zachqieja, J.J., 1998. A leptin dose-response study in obese (ob/ob) and lean (+/?) mice. Endocrinology 139, 8–19.

Hart, J.S., 1950. Interrelations of daily metabolic cycle, activity and environmental temperature of mice. Can. J. Res. 28, 293–307.

Herrington, L.P., 1940. The heat regulation of small laboratory animals at various environmental temperatures. Am. J. Physiol. 129, 123–129.

Hogan, S., Himms-Hagen, J., 1980. Abnormal brown adipose tissue in obese (ob/ob) mice: response to acclimation to cold. Am. J. Physiol. 239, E301–E309.

Hogan, E., Costantini, F., Lacy, E., 1986. Manipulating the Mouse Embryo. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Horai, R., Asano, M., Sudo, K., Kanuka, H., Suzuki, M., Nishihara, M., Takahashi, M., Iwakura, Y., 1998. Production of mice deficient in genes for interleukin (IL)-1α, IL-1β, IL-1α/β, and IL-1 receptor antagonist shows that IL-1β is crucial in turpentine-induced fever development and glucocorticoid secretion. J. Exp. Med. 187, 1463–1475.

Hudson, J.W., Scott, I.M., 1979. Daily torpor in the laboratory mouse, *Mus musculus*, var. albino. Physiol. Zool. 52, 205–218.

Ingalls, A.M., Dickie, M.M., Snell, G.D., 1950. Obese, a new mutation in the house mouse. J. Hered. 41, 317–318.

IUPS, 2001. Glossary of terms for thermal physiology. Jpn. J. Physiol. 51, 245–280.

Justice, M.J., Noveroske, J.K., Weber, J.S., Zheng, B., Bradley, A., 1999. Mouse ENU mutagenesis. Hum. Mol. Genet. 8, 1955–1963.

Kluger, M.J., 1991. Fever: role of pyrogens and cryogens. Physiol. Rev. 71, 93–127.

Kozak, W., Conn, C.A., Kluger, M.J., 1994. Lipopolysaccharide induces fever and depresses locomotor activity in unrestrained mice. Am. J. Physiol. 266, R125–R135.

Kozak, W., Conn, C.A., Klir, J.J., Wong, G.W.H., Kluger, M.J., 1995. TNF soluble receptor and antiserum against TNF enhance lipopolysaccharide fever in mice. Am. J. Physiol. 269, R23–R29.

Kozak, W., Kluger, M.J., Soszynski, D., Conn, C.A., Rudolph, K., Leon, L.R., Zheng, H., 1998. IL-6 and IL-1β in fever. Studies using cytokine-deficient (knockout) mice. Ann. N.Y. Acad. Sci. 856, 33–47.

Kuhn, R., Schwenk, F., Aguet, M., Rajewsky, K., 1995. Inducible gene targeting in mice. Science 269, 1427–1429.

Labow, M., Shuster, D., Zetterstrom, M., Nunes, P., Terry, R., Cullinan, E.B., Bartfai, T., Solorzano, C., Moldawer, L.L., Chizzonite, R., McIntyre, K.W., 1997. Absence of IL-1 signaling and reduced inflammatory response in IL-1 type I receptor-deficient mice. J. Immunol. 159, 2452–2461.

Lathe, R., 1996. Mice, gene targeting and behaviour: more than just genetic background. Trends Neurosci. 19, 183–186.

Leon, L.R., Conn, C.A., Glaccum, M., Kluger, M.J., 1996. IL-1 type I receptor mediates acute phase response to turpentine, but not lipopolysaccharide, in mice. Am. J. Physiol. 271, R1668–R1675.

Leon, L.R., DuBose, D.A., Walker, L.D., 2004. Editorial focus: biotelemetry transmitter implantation in rodents: impact on growth and circadian rhythms. Am. J. Physiol. 286, R967–R974.

Luheshi, G., Miller, A.J., Brouwer, S., Dascombe, M.J., Rothwell, N.J., Hopkins, S.J., 1996. Interleukin-1 receptor antagonist inhibits endotoxin fever and systemic interleukin-6 induction in the rat. Am. J. Physiol. 270, E91–E95.

Markel, P., Shu, P., Ebeling, C., Carlson, G.A., Nagle, D.L., Smutko, J.S., Moore, K.J., 1997. Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains. Nat. Genet. 17, 280–284.

Melton, D.W., 1994. Gene targeting in the mouse. BioEssays 16, 633–638.

Meyers, E.N., Lewandoski, M., Martin, G.R., 1998. An Fgf8 mutant allele series generated by Cre- and FLP-mediated recombination. Nat. Genet. 18, 136–141.

Moore, R.C., Redhead, N.J., Selfridge, J., Hope, J., Manson, J.C., Melton, D.W., 1995. Double replacement gene targeting for the production of a series of mouse strains with different prion protein gene alterations. Biotechnology 13, 999–1004.

Morimoto, A., Murakami, N., Nakamori, T., Watanabe, T., 1986. Suppression of nonshivering thermogenesis in the rat by heat-seeking behavior during cold exposure. J. Physiol. 380, 541–549.

Mount, L.E., 1971. Metabolic rate and thermal insulation in albino and hairless mice. J. Physiol. Lond. 217, 315–326.

Muller, U., 1999. Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. Mech. Dev. 82, 3–21.

Noveroske, J.K., Weber, J.S., Justice, M.J., 2000. The mutagenic action of *N*-ethyl-*N*-nitrosourea in the mouse. Mamm. Genome 11, 478–483.

Ogilvie, D.M., Stinson, R.H., 1966. The effect of age on temperature selection by laboratory mice (*Mus musculus*). Can. J. Zool. 44, 511–517.

Ohtake, M., Bray, G.A., Azukizawa, M., 1977. Studies on hypothermia and thyroid function in the obese (ob/ob) mouse. Am. J. Physiol. 233, R110–R115.

Oka, T., Oka, K., Kobayashi, T., Sugimoto, Y., Ichikawa, A., Ushikubi, F., Narumiya, S., Saber, C.B., 2003. Characteristics of thermoregulatory and febrile responses of mice deficient in EP1 and EP3 receptors. J. Physiol. 551, 945–954.

Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., Collins, F., 1995. Effects of the *obese* gene product on body weight regulation in ob/ob mice. Science 269, 540–543.

Poon, A.M.S., Wu, B.M., Poon, P.W.F., Cheung, E.P.W., Chan, F.H.Y., Lam, F.K., 1997. Effect of cage size on ultradian locomotor rhythms of laboratory mice. Physiol. Behav. 62, 1253–1258.

Printz, M.P., 2004. Radiotelemetry comes of age—perhaps just in time. Am. J. Physiol. 286, R818–R819.

Qiu, J., Ogus, S., Mounzih, K., Ewart-Toland, A., Chehab, F.F., 2001. Leptin-deficient mice backcrossed to the BALB/cJ genetic background have reduced adiposity, enhanced fertility, normal body temperature, and severe diabetes. Endocrinology 142, 3421–3425.

Rajewsky, K., Gu, H., Kuhn, R., Betz, U.A.K., Muller, W., Roes, J., Schwenk, F., 1996. Moledular medicine in genetically engineered animals. J. Clin. Invest. 98, 600–603.

Refinetti, R., 1997. The effects of ambient temperature on the body temperature rhythm of rats, hamsters, gerbils, and tree shrews. J. Therm. Biol. 22, 281–284.

Rijli, F.M., Dolle, P., Fraulob, V., LeMeur, M., Chambou, P., 1994. Insertion of a targeting construct in a Hoxd-10 allele can influence the control of Hoxd-9 expression. Dev. Dyn. 201, 366–377.

Schmidt, I., 1984. Interaction of Behavioural and Autonomic Thermoregulation. Raven Press, New York.

Schobitz, B., Pezeshki, G., Pohl, T., Hemmann, U., Heinrich, P.C., Holsboer, F., Reul, J.M.H.M., 1995. Soluble interleukin-6 (IL-6) receptor augments central effects of IL-6 in vivo. FASEB J. 9, 659–664.

Seibler, J., Zevnik, B., Kuter-Luks, B., Andreas, S., Kern, H., Hennek, T., Rode, A., Heimann, C., Faust, N., Kauselmann, G., Schoor, M., Jaenisch, R., Rajewsky, K., Kuhn, R., Schwenk, F., 2003. Rapid generation of inducible mouse mutants. Nucleic Acids Res. 31, 1–8.

Sigmund, C.D., 1993. Major approaches for generating and analyzing transgenic mice. Hypertension 22, 599–607.

Sigmund, C.D., 2000. Viewpoint: are studies in genetically altered mice out of control? Arterioscler Thromb. Vasc. Biol. 20, 1425–1429.

Silva, A.J., 1997. Mutant mice and neuroscience: recommendations concerning genetic background. Banbury conference on genetic background in mice. Neuron 19, 755–759.

Simpson, E.M., Linder, C.C., Sargent, E.E., Davisson, MT., Mobraaten, L.E., Sharp, J.J., 1997. Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. Nat. Genet. 19–27.

Sims, J.E., Gayle, M.A., Slack, J.L., Alderson, M.R., Bird, T.A., Giri, J.G., Colotta, F., Re, F., Mantovani, A., Shanebeck, K., Grabstein, K.H., Dower, S.K., 1993. Interleukin-1 signaling occurs exclusively via the type I receptor. Proc. Natl. Acad. Sci. USA 90, 6155–6159.

Smits, B.M.G., Mudde, J., Plasterk, R.H.A., Cuppen, E., 2004. Target-selected mutagenesis of the rat. Genomics 83, 332–334.

Stoen, R., Sessler, D.I., 1990. The thermoregulatory threshold is inversely proportional to isoflurane concentration. Anesthesiology 72, 822–827.

Swoap, S.J., Overton, J.M., Garber, G., 2004. Effect of ambient temperature on cardiovascular parameters in rats and mice: a comparative approach. Am. J. Physiol. 287, R391–R396.

Szelenyi, Z., Hummel, Z., Szolcsanyi, J., Davis, J.B., 2004. Daily body temperature rhythm and heat tolerance in TRPV1 knockout and capsaicin pretreated mice. Eur. J. Neurosci. 19, 1421–1424.

Taketo, M., Schroeder, A.C., Mobraaten, L.E., Gunning, K.B., Hanten, G., Fox, R.R., Roderick, T.H., Stewart, C.L., Lilly, F., Hansen, C.T., Overbeek, P.A., 1991. FVB/N: an inbred mouse strain preferable for transgenic analysis. Proc. Natl. Acad. Sci. USA 88, 2065–2069.

Threadgill, D.W., Yee, D., Matin, A., Nadeau, J.H., Magnuson, T., 1997. Genealogy of the 129 inbred strains: 129/SvJ is a contaminated inbred strain. Mamm. Genome 8, 390–393.

Thyagarajan, T., Totey, S., Danton, M.J.S., Kulkarni, A.B., 2003. Genetically altered mouse models: the good, the bad, and the ugly. Crit. Rev. Oral. Biol. Med. 14, 154–174.

Trayhurn, P., James, W.P.T., 1978. Thermoregulation and non-shivering thermogenesis in the genetically obese (ob/ob) mouse. Pflugers Arch. 373, 189–193.

Trayhurn, P., Thurlby, P.L., James, W.P.T., 1976. A defective response to cold in the obese (ob/ob) mouse and the obese Zucker (fa/fa) rat. Proc. Nutr. Soc. 35, 133A.

Van der Weyden, L., Adams, D.J., Bradley, A., 2002. Tools for targeted manipulation of the mouse genome. Physiol. Genomics 11, 133–164.

Wakeland, E., Morel, L., Achey, K., Yui, M., Longmate, J., 1997. Speed congenics: a classic technique in the fast lane (relatively speaking). Immunol. Today 18, 472–477.

Wang, Y., Spatz, M.K., Kannan, K., Hayk, H., Avivi, A., Gorivodsky, M., Pines, M., Yayon, A., Lonai, P., Givol, D., 1999. A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. Proc. Natl. Acad. Sci. USA 1, 4455–4460.

Weigle, D.S., Bukowski, T.R., Foster, D.C., Holderman, S., Kramer, J.M., Lasser, G., Lofton-Day, C.E., Prunkard, D.E., Raymond, C., Kuijper, J.L., 1995. Recombinant ob protein reduces feeding and body weight in the *ob/ob* mouse. J. Clin. Invest. 96, 2065–2070.

Weinert, D., Waterhouse, J., 1998. Diurnally changing effects of locomotor activity on body temperature in laboratory mice. Physiol. Behav. 63, 837–843.

Wilder, P.J., Rizzino, A., 1993. Mouse genetics in the 21st century: using gene targeting to create a cornucopia of mouse mutants possessing precise genetic modifications. Cytotechnology 11, 79–99.

Wilson, L.M., Sinha, H.L., 1985. Thermal preference behavior of genetically obese (*ob/ob*) and genetically lean (+/−) mice. Physiol. Behav. 35, 545–548.

Yap, G.S., Sher, A., 2002. The use of germ line-mutated mice in understanding host-pathogen interactions. Cell. Microbiol. 4, 627–634.

Zan, Y., Haag, J.D., Chen, K.-S., Shepel, L.A., Wigington, D., Wang, Y.-R., Hu, R., Lopez-Guajardo, C.C., Brose, H.L., Porter, K.I., Leonard, R.A., Hitt, A.A., Schommer, S.L., Elegbede, A.F., Gould, M.N., 2003. Production of knockout rats using ENU mutagenesis and a yeast-based screening assay. Nat. Biotech. 21, 645–651.

Zhang, Y., Porenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M., 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372, 425–432.

Zheng, H., Fletcher, D., Kozak, W., Jiang, M., Hofmann, K.J., Conn, C.A., Soszynski, D., Grabiec, C., Trumbauer, M.E., Shaw, A., Knockoutstura, M.J., Stevens, K., Rosen, H., North, R.J., Chen, H.Y., Tocci, M.J., Kluger, M.J., Van der Ploeg, L.H.T., 1995. Resistance to fever induction and impaired acute-phase response in interleukin-1β-deficient mice. Immunity 3, 9–19.

Zou, Y.R., Muller, W., Gu, H., Rajewsky, K., 1994. Cre-loxP-mediated gene replacement: a mouse strain producing humanized antibodies. Curr. Biol. 4, 1099–1203.